Original Research

Molecular biomarker panels for assessment of selenium status in rats

Roger A Sunde

Department of Nutritional Sciences, University of Wisconsin, 1415 Linden Drive, Madison, WI 53706, USA
Corresponding author: Roger A Sunde. Email: sunde@nutrisci.wisc.edu

Abstract

Molecular biomarkers are mRNA transcripts that indicate the (nutrient) status of an organism or tissue. Molecular biomarker panels have the potential to readily and more accurately determine nutrient status than individual traditional biomarkers. To study the efficacy of molecular biomarker panels for predicting selenium (Se) status, we examined 30 biomarkers from rats fed graded levels of Se from deficient to eight times the minimum Se requirement, including four liver and four kidney traditional biomarkers, and 13 liver and nine kidney selenoprotein mRNA levels. Multiple regression analysis against liver and kidney Se and glutathione peroxidase-1 (Gpx1) activity, with stepwise single elimination of biomarkers that did not significantly contribute, was used to identify biomarker panels with significant ($P < 0.05$) regression coefficients. Resulting regression equations were then used to predict Se status, and compared with traditional Se biomarkers panels. Over the full spectrum of Se status from 0 to 0.8 μg Se/g diet, the resulting 4-selenoprotein mRNA biomarker panel predicted liver Se concentration with a correlation of 0.948, which was nominally higher and statistically the same as the correlation of 0.909 for the panel based on Gpx1 activity. The molecular biomarker panels for predicting kidney Se and liver and kidney Gpx1 activity were all comparable to predictions based on traditional biomarkers. These analyses show that molecular biomarker panels can be used to predict accurately two traditional biomarkers of Se status. The resulting analyses also illustrate that additional orthogonal biomarkers reflecting higher Se intakes are needed to better predict supernutritional Se status and further strengthen this approach.

Keywords: glutathione peroxidase, kidney, liver, mRNA, nutrition, panel, requirements

Experimental Biology and Medicine 2010; **235**: 1046–1052. DOI: 10.1258/ebm.2010.010111

Introduction

A biomarker is a measurable quantity that can serve as an indicator of normal biological or pathological processes;[1] a molecular biomarker can be defined as an mRNA transcript that indicates the (nutrient) status of an organism or tissue, as distinguished from biochemical biomarkers, such as enzyme activity, or chemical biomarkers, such as the concentration of an element, vitamin or metabolite.[2] Invariably, recent reports on nutrient status and requirements, or on multivitamin and mineral supplement use, have called for new biomarkers for both deficiency and toxicity for nearly all essential nutrients, and for biomarkers that will be useful early in disease development.[3,4] Advances in genomics, biotechnology and bioinformatics are providing new molecular biomarker candidates at an accelerating pace, which are likely to have efficacy in both medicine and nutrition for early screening and diagnosis, and for monitoring of therapeutic treatments.[2]

An important aspect of biomarker development is the identification of panels of biomarkers that can be used together to provide more accurate and robust predictors of a patient's condition and optimum treatment than are provided by individual biomarkers.[5] There are over 500 reports in the literature on development of biomarker panels using mRNA transcripts in areas such as medicine, toxicology, and cancer diagnosis and treatment, but not one of these reports is on molecular biomarker panels for assessment of status of an essential nutrient.[2]

Selenium (Se) status regulates the expression of glutathione peroxidase-1 (Gpx1) mRNA as well as activity,[6] and we have used mRNA levels of Gpx1[7–10] and other selenoproteins[11,12] to determine Se status and requirements in rats. In humans, so far only modest changes in a few mRNA transcripts in response to Se supplementation have been reported.[13–16] In rats, mRNA levels for a number of zinc transporters as well as metallothionein are altered by zinc status,[17,18] and in humans, several groups have shown that changes in zinc intake alter blood mRNA levels for metallothionein and two zinc transporters.[19–21] Lastly, a quantitative trait loci approach compared

differences in hippocampus iron, copper and zinc concentrations with gene expression databases for different mouse strains and identified 27 genes whose expression was related to brain metal concentrations.[22] In none of these human or animal nutrition studies, however, have the data been used to develop molecular biomarker panels for calculation of tissue concentration or nutrient-dependent enzyme activity.

Our recent rat study,[11] with graded levels of dietary Se ranging from deficient to supernutritional (eight times requirement), determined both traditional biomarkers of Se status (Se and selenoenzyme activities in liver and kidney) and relative levels of 13 selenoprotein mRNA transcripts in the liver and nine in the kidney. These biomarkers are the data-sets we studied here to demonstrate the efficacy of molecular biomarker panels to predict Se status. The hypothesis was that multiple regression analysis could be used to develop molecular biomarker panels for predicting Se concentration and Gpx1 activity in the liver and kidney that would be equivalent to biomarker panels based on traditional biomarkers.

Materials and methods

Animals and diets

Male weanling rats (21 d old) were obtained from Holtzman (Madison, WI, USA) and housed individually in hanging wire-mesh cages, as described previously in detail.[11] Rats were fed the basal Se-deficient diet (0.005 μg Se/g by analysis) supplemented with graded levels of Se (0, 0.016, 0.04, 0.06, 0.08, 0.12, 0.16, 0.24, 0.4 or 0.8 μg Se/g as Na_2SeO_3) for 28 d (3 rats/treatment). Animals had free access to feed and water, and the care and treatment protocols were approved by the Institutional Animal Care and Use Committee. Body weight was measured bi-weekly, and there was no effect of dietary Se on growth.[11]

Traditional Se biomarkers

Rats were anesthetized with ether, and blood was drawn by cardiac puncture using EDTA-containing syringes. Liver and kidney Se concentrations were determined by neutron activation analysis.[23] Gpx1 activity in the liver and kidney (designated as Gpx1) and Gpx3 activity in the plasma (designated as Gpx3) were measured by the coupled assay procedure,[24] and phospholipid hydroperoxide Gpx (Gpx4) activity was measured[7] using 78 μmol/L phosphatidylcholine hydroperoxide. Liver thioredoxin reductase (Txnrd) was measured using the gold-inhibition assay with 5,5′-dithio-bis (2-nitrobenzoic acid) as a substrate.[25]

Selenoprotein mRNA transcript biomarkers

Total RNA from the liver and kidney ($n = 3$/diet group) was isolated and analyzed as described previously.[11] Relative mRNA abundance was determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR) using gene-specific primers as described previously,[11] normalized to the mean of β-actin and glyceraldehyde-3-phosphate dehydrogenase mRNAs, and expressed as a percentage of Se-adequate plateau levels.[11]

Statistical analysis

Initially, individual values for each biomarker were rescaled linearly on a range between 0 and 10, and then the data-sets were subjected to single and multiple regression analysis, as described by Steele and Torrie,[26] using SPSS (v15; SPSS Inc, Chicago, IL, USA). Data-sets were regressed against the corresponding data-sets for liver Se and Gpx1 activity or for kidney Se and Gpx1 activity to determine correlation coefficients (r), regression coefficients (β) and significance (P value). Multiple regression analysis was conducted with each data-set (4 traditional liver biomarkers, 13 molecular liver biomarkers, 4 traditional kidney biomarkers or 9 molecular kidney biomarkers; see Table 1). To identify a

Table 1 Single linear regression analysis of Se biomarkers

	Versus Se conc.		Versus Gpx1 act.	
	r	*P* value	*r*	*P* value
Liver biomarkers				
Traditional				
Se conc.	1.000	–	0.909	3.99E−12
Gpx1 act.	0.909	3.99E−12	1.000	–
Gpx4 act.	0.801	1.09E−07	0.799	1.17E−07
Txnrd act.	0.879	1.71E−10	0.916	1.26E−12
Molecular				
Gpx1 mRNA	0.866	6.10E−10	0.904	7.76E−12
Gpx3 mRNA	0.568	1.05E−03	0.313	9.24E−02
Selt mRNA	0.582	7.49E−04	0.620	2.62E−04
Selk mRNA	0.686	2.87E−05	0.706	1.29E−05
Selh mRNA	0.799	1.21E−07	0.774	5.28E−07
Sep15 mRNA	0.666	5.97E−05	0.691	2.35E−05
Sepn1 mRNA	0.544	1.89E−03	0.652	9.57E−05
Sepp1 mRNA	0.666	5.91E−05	0.679	3.69E−05
Sepw1 mRNA	0.803	9.42E−08	0.807	7.41E−08
Txnrd3 mRNA	0.676	4.10E−05	0.659	7.45E−05
Dio1 mRNA	0.631	1.88E−04	0.678	3.81E−05
Sephs2 mRNA	0.265	1.57E−01	0.318	8.65E−02
Gpx4 mRNA	0.470	8.82E−03	0.386	3.51E−02
Kidney biomarkers				
Traditional				
Se conc.	1.000	–	0.903	8.25E−12
Gpx1 act.	0.903	8.25E−12	1.000	–
Gpx4 act.	0.644	1.22E−04	0.672	4.85E−05
Plasma Gpx3 act.	0.877	2.06E−10	0.954	3.47E−16
Molecular				
Gpx1 mRNA	0.702	1.51E−05	0.800	1.12E−07
Gpx4 mRNA	0.002	9.90E−01	0.064	7.37E−01
Gpx3 mRNA	0.576	8.58E−04	0.683	3.21E−05
Selh mRNA	0.732	4.37E−06	0.795	1.54E−07
Selk mRNA	0.748	2.06E−06	0.741	2.85E−06
Sepn1 mRNA	0.526	2.83E−03	0.483	6.91E−03
Sepw1 mRNA	0.779	3.99E−07	0.839	6.75E−09
Txnrd1 mRNA	0.551	1.61E−03	0.743	2.56E−06
Sephs2 mRNA	−0.533	2.44E−03	−0.521	3.13E−03

Gpx1, glutathione peroxidase-1; Gpx4, phospholipid hydroperoxide Gpx; Se, selenium; Txnrd, thioredoxin reductase; Selt, selenoprotein T; Selk, selenoprotein K; Selh, selenoprotein H; Sepw1, selenoprotein W
Correlation coefficients (r) and significance (P value) for single regression analysis of the indicated biomarker versus Se concentration or glutathione peroxidase activity (Gpx1 Act.) in the liver (top) or kidney (bottom)

significant biomarker panel, data for the biomarker with the least significant coefficient (highest P value) were removed from the data-set, in a stepwise manner, and the multiple regression repeated, until each biomarker regression coefficient was significant ($P < 0.05$). At this point, the original data were reanalyzed using Excel 2007 with the Data Analysis add-in (Microsoft) to confirm the analyses and calculate regression coefficients reflecting the original (un-rescaled) values. The resulting multiple regression equations using these significant coefficients were then used to calculate the dependent variable (tissue Se or Gpx1 activity) for each individual rat based on the measured biomarkers. Fisher's z-test[26] was used to compare correlation coefficients. For all tests, $P < 0.05$ was considered significant.

Results

The 30 biomarkers examined here (listed in Table 1) were used by Barnes *et al.*[11] to generate Se response curves and to calculate plateau breakpoints and determine minimum dietary Se requirements for these biomarkers. The resulting minimum dietary Se requirements determined using highly regulated biomarkers ranged from 0.06 to 0.12 μg Se/g for traditional biomarkers and 0.03–0.07 μg Se/g for molecular biomarkers in the liver and kidney, as reported previously.[11] Single linear regression analysis of each biomarker set against the respective Se concentration or Gpx1 activity are shown in Table 1. Traditional liver biomarker correlation coefficients were highly significant and between 0.801 and 0.909 for liver Se, and between 0.799 and 0.916 for liver Gpx1 activity. The traditional kidney biomarkers included plasma Gpx3 activity because kidney is the major organ for plasma Gpx3 synthesis.[27] For kidney, correlations were highly significant and between 0.644 and 0.903 for kidney Se, and between 0.672 and 0.954 for kidney Gpx1 activity. In this study, we previously reported that transcripts for Gpx1, selenoprotein W (Sepw1), selenoprotein H and selenoprotein K (Selk) were highly regulated by dietary Se.[11] For these molecular biomarkers in the liver, correlations were highly significant and between 0.686 and 0.866 against liver Se, and between 0.706 and 0.904 against liver Gpx1 activity (Table 1). For these molecular biomarkers in the kidney, correlations were highly significant and between 0.702 and 0.779 against kidney Se, and between 0.741 and 0.839 against kidney Gpx1 activity. Other selenoprotein mRNA transcripts, however, were far less correlated with tissue Se and Gpx1 activity, including kidney Sephs2, which was inversely correlated, as we reported in the original publication,[11] and liver Sephs2, which was not significantly correlated ($P > 0.05$). Gpx4 transcripts, representative of the majority of selenoprotein mRNAs, which are not significantly regulated by dietary Se,[11] were also not significantly correlated with liver Gpx1 activity, kidney Gpx1 activity or kidney Se.

Multiple regression analysis with the full biomarker data-set against liver Se resulted in a correlation of 0.918 for the traditional biomarkers and 0.958 for the molecular biomarkers (Table 2). Stepwise removal of biomarkers with non-significant coefficients ($P > 0.05$) from the analysis resulted in a 1-enzyme traditional biomarker panel with a correlation coefficient of 0.909, and resulted in a 4-mRNA molecular biomarker panel with a correlation coefficient of 0.948; these two correlation coefficients are not significantly different ($P = 0.28$), indicating that this biomarker panel is just as effective as the traditional biomarker panel. These correlation coefficients indicate that these regressions account for 83% and 90%, respectively, of the liver Se concentrations. Corresponding regression equation coefficients are given in Table 3.

Table 2 Multiple regression correlation coefficients

	Se concentration		Gpx1 activity	
	All	Panel	All	Panel
Liver panels				
Traditional	0.918 (3)	0.909* (1)	0.943 (3)	0.942* (2)
Molecular	0.958 (13)	0.948* (4)	0.945 (13)	0.924* (2)
Kidney panels				
Traditional	0.906 (3)	0.903* (1)	0.965 (3)	0.964* (2)
Molecular	0.880 (9)	0.839* (2)	0.938 (9)	0.914* (3)

Gpx1, glutathione peroxidase-1; Se, selenium
Correlation coefficients (r) of multiple regression analyses using all biomarkers (All) or the panel biomarkers (biomarkers with significant regression coefficients included in final panels) versus Se concentration or glutathione peroxidase activity in the liver (top) or kidney (bottom). Values in parentheses indicate the number of biomarkers included in each analysis. Shown multiple regressions are all highly significant ($P < 0.0001$). Asterisks (*) indicate that the correlation coefficients for traditional versus molecular biomarkers panels were not significantly different ($P > 0.05$)

The biomarker panel analysis against liver Gpx1 activity resulted in a traditional 2-biomarker panel, with a correlation coefficient of 0.942, and in a 2-mRNA molecular biomarker panel, with a correlation coefficient of 0.924 (Table 2). Similar multiple regression analysis for kidney Se resulted in a 1-enzyme traditional biomarker panel with a correlation coefficient of 0.903, and resulted in a 2-mRNA molecular biomarker panel with a correlation coefficient of 0.839; for kidney Gpx1 activity, analysis resulted in a traditional 2-biomarker panel with a correlation coefficient of 0.964, and resulted in a 3-mRNA molecular biomarker panel with a correlation coefficient of 0.914.

Figure 1 shows the liver and kidney Se concentrations and Gpx1 activities for the 30 rats calculated from these regression equations and plotted as group means. Measured mean actual Se concentrations and Gpx1 activities are shown for comparison. Clearly, the resulting molecular biomarker panels readily predict levels in the liver and kidney. Reasons as to why the composition of the final panels differs for liver and kidney or for Se concentration versus Gpx1 activity are unclear, but likely reflect differences in Se distribution and selenoprotein expression in these two organs.

Discussion

The development of molecular biomarker panels, using stepwise multiple regression to eliminate biomarkers that do not significantly contribute, was used to identify molecular biomarker panels for the assessment of Se status (Figure 1). Over the full spectrum of Se status from deficient

Table 3 Biomarker panel multiple regression coefficients

Traditional panels				Molecular panels			
Biomarker	β	**SE**	***P* value**	**Biomarker**	β	**SE**	***P* value**
Liver Se							
Gpx1 act.	0.00982	0.000085	<0.0001	Gpx1 mRNA	11.91	1.40	<0.0001
Intercept	0.493	0.559	0.385	Gpx3 mRNA	4.90	0.96	<0.0001
				Selt mRNA	−3.40	1.38	0.021
				Selk mRNA	−3.88	1.75	0.036
				Intercept	−0.98	1.12	0.391
Liver Gpx1 act.							
Se conc.	42.05	12.58	0.0024	Gpx1 mRNA	1133.4	139.5	<0.0001
Txnrd act.	17.14	4.51	<0.0008	Selk mRNA	−476.0	180.4	0.0137
Intercept	−102.26	59.12	0.0951	Intercept	32.30	87.79	0.716
Kidney Se							
Gpx1 act.	0.0271	0.0024	<0.0001	Sepw1 mRNA	9.13	2.51	0.0012
Intercept	1.589	0.864	0.0764	Selk mRNA	14.17	4.76	0.0061
				Intercept	−10.76	3.12	0.0018
Kidney Gpx1							
Pl. Gpx3 act.	3.82	0.58	<0.0001	Sepw1 mRNA	302.0	66.3	0.0001
Se conc.	9.65	3.55	0.0113	Txnrd1 mRNA	299.4	140.5	0.0427
Intercept	−41.48	20.70	0.0552	Gpx1 mRNA	1.9	0.8	0.0294
				Intercept	−334.6	79.6	0.0003

Gpx1 act., glutathione peroxidase activity; Gpx3, plasma GPX; Se conc., Se concentration; Selt, selenoprotein T; Selk, selenoprotein K; Sepw1, selenoprotein W; Txnrd, thioredoxin reductase

Regression coefficients (β), standard errors (SE) and significance (*P* value) from multiple regression analyses on panel biomarkers (biomarkers with significant regression coefficients) versus Se conc. or Gpx1 act. in the liver or kidney, based on enzyme biomarker panels (left) or molecular biomarker panels (right). Regression equations are of the form: $Y = \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \ldots + \varepsilon$, where Y is the calculated dependent variable, β_i are the significant regression coefficients, X_i are the individual biomarker values and ε is the intercept (error term). Shown multiple regressions are all highly significant ($P < 0.0001$)

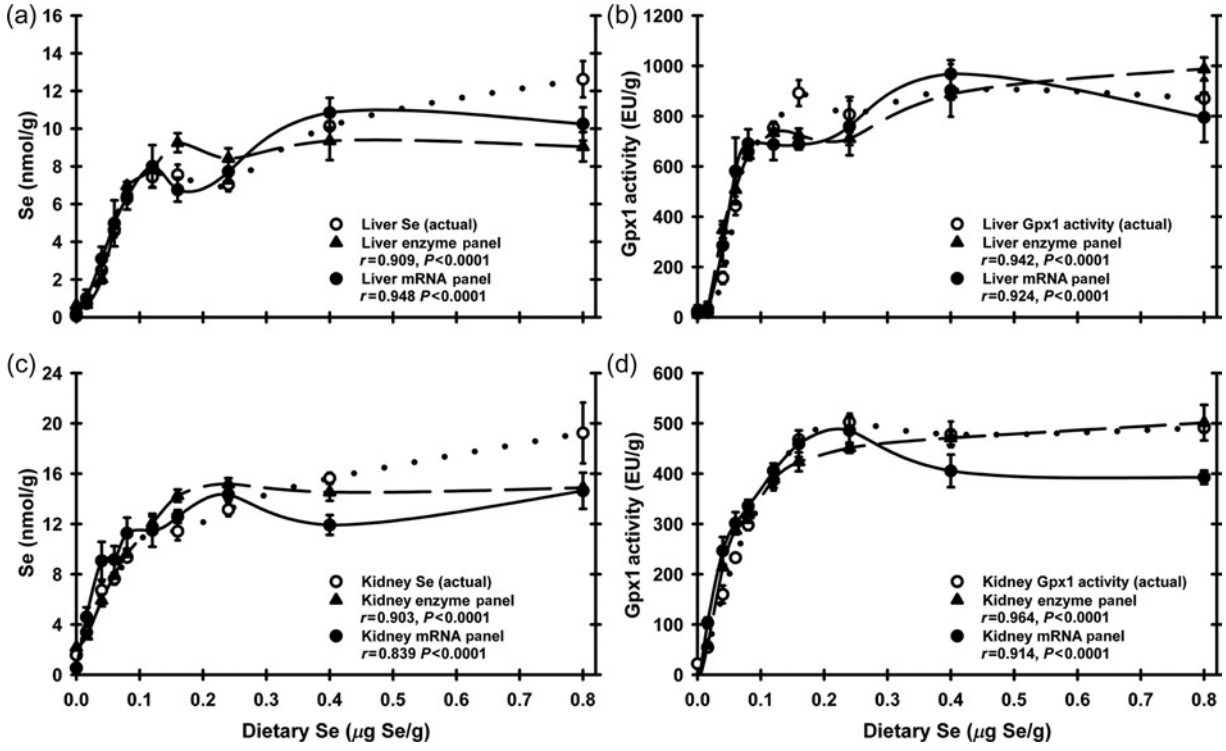

Figure 1 Se concentrations and glutathione peroxidase-1 (Gpx1) activities as determined by actual measurement or calculated using biomarker panel multiple regression equations (see Table 3). Rats were fed indicated levels of dietary selenium (Se), and Se concentrations, selenoenzyme activities and selenoprotein mRNA levels were determined in the liver, kidney and plasma. Values are means ± SEM ($n = 3$/dietary treatment). Multiple regression analysis was conducted to identify biomarkers with significant ($P < 0.05$) coefficients, which were then used to generate biomarker panels and equations for calculation of Se concentration or Gpx1 activity, as described in the text. Shown are Se concentrations (a, c) and Gpx1 activities (b, d) in the liver (a, b) and kidney (c, d), as calculated by these traditional (Enzyme) panels or mRNA-based (Molecular) biomarker panels. Correlation coefficients (*r*) are indicated; all multiple regressions are highly significant ($P < 0.0001$)

to eight times the minimal Se requirement, the liver Se concentrations calculated with the molecular biomarker panel had a correlation coefficient with measured Se levels that were nominally higher and statistically the same as the correlation determined with the traditional selenoenzyme-based panel. For kidney Se and for liver and kidney Gpx1, the values predicted by the molecular biomarker panels were also well correlated with measured values, and also essentially the same as the values predicted with the traditional biomarker-based panels. These resulting Se response curves clearly show that these molecular biomarker panels can be used to identify rats with deficient Se status.

Using these sets of candidate biomarkers, this analysis illustrates a process for identifying molecular biomarker panels for estimating nutrient status, and it illustrates that the predicted markers of Se status and actual measured values can be in high agreement. This is not at all surprising for a biomarker panel using Gpx1 activity to estimate Se concentration, as ~75% of Se in Se-adequate liver is present as Se in Gpx1.[28] What is far more remarkable is that a similar relationship holds for the relationship of liver Gpx1, Gpx3, selenoprotein T (Selt) and Selk mRNAs, which do not directly contribute Se to the overall liver Se content, but rather provide templates for the synthesis of liver selenoproteins.

The ability of these mRNA transcript-based biomarker panels to predict tissue Se or Gpx1 activity is perhaps also not unexpected as we found that transcript levels for all of these selenoprotein mRNAs in the liver and kidney decreased highly and significantly in Se deficiency and plateaued by 0.1 μg Se/g diet.[11] The good agreement shown in Figure 1, however, is worth noting for two points. First, the multiple regression analyses were conducted not only just over the range between Se deficient and the Se requirement (0.1 μg Se/g), but also included data for up to eight times the requirement. The resulting regressions thus predict rather accurately the measured values. If instead, the multiple regression analysis was conducted only over the 0–0.12 μg Se/g range, the resulting regression actually includes more significant biomarkers, but also grossly miss-estimates Se status in rats fed levels outside the range used for the analysis. For example, use of this narrow-Se-range panel to predict liver Se in rats fed 0.8 μg Se/g diet results in a prediction threefold above the measured level (data not shown). This illustrates that multiple regression analysis should include the full expected range of nutrient status to accurately predict status. Second, the essentially flat predicted Se response curves (Figure 1) after ~0.2 μg Se/g diet, as compared with the continued but slow further increase in liver and kidney Se, clearly illustrate that additional, orthogonal biomarkers for supernutritional status are needed to better predict this increase in tissue Se. Unfortunately, there are no good biomarkers for high Se intake.[3,29] These biomarkers are especially needed because higher intakes of Se are associated with prevention of cancer and other diseases,[30–32] and yet recommending optimum Se intake levels is now further complicated because a number of recent original studies and meta-analyses indicate that Se supplementation may have adverse effects on human health.[33–38]

A number of similar approaches are now reported for applications in medicine, cancer or toxicology. Dakashita *et al.*[39] used microarray analysis of 20 human subjects in a cadmium-polluted area, where median blood and urinary cadmium concentrations were 2.4 and 1.9 times higher than in a non-cadmium-polluted area, and compared this group versus gender- and age-matched subjects in the non-cadmium-polluted area. RNA isolated from total blood was subjected to microarray analysis using arrays designed to detect transcripts of 1467 genes related to stress response plus 400 transcripts related to drug metabolism; 137 upregulated and 80 downregulated transcripts were detected. Changes were correlated with cadmium levels in blood and urine, best correlated transcripts confirmed by quantitative RT-PCR and multiple regression analysis of 17 transcripts was used to identify nine genes with significant regression coefficients. After culling due to adjustment for hypertension and diabetes, this resulted in a molecular biomarker panel of seven transcripts for detection of chronic low level cadmium exposure. Similar approaches include several reports of molecular biomarker panels to identify cancer cells in peripheral blood of patients with colorectal cancer[40] or breast cancer,[41] or molecular biomarker panels on tumor samples to predict lymph node involvement in gastric cancer.[42] These applications in toxicology or cancer detection illustrate the potential of the approach in nutrition.[2]

In 1988, we discovered that Se status regulates the expression of Gpx1 mRNA as well as activity,[6] and then used this regulation to determine Se status and requirements in rats.[7–9] This molecular biomarker approach was especially helpful in determining Se requirements in pregnant and lactating rats as it illustrates that the nutritionally adequate level of a biomarker can change substantially over the life cycle.[10] More recently, we conducted a microarray study to examine the Se regulation of the full selenoproteome and found other selenoprotein mRNAs that are highly regulated by Se status.[11,43] Our search for surrogate molecular biomarkers now extends to RNA readily isolated from whole blood, where Gpx1 mRNA is expressed at levels comparable to liver and kidney,[44] and can be used to assess Se status and requirements in rats.[45] Our attempt to extend this approach to a human population in Reading, UK, consuming an average of 48 μg Se/d, found, however, that Se status was not sufficiently low to detect significant changes in mRNA transcripts,[16] which were Se regulated in rodent studies. These studies do show that molecular biomarkers have the potential for assessment of Se status.[2]

In summary, this analysis illustrates that stepwise multiple regression, to eliminate biomarkers that do not significantly contribute, can be used effectively to identify molecular biomarker panels for assessment of Se status. The resulting regression equations accurately predict tissue Se concentration and Gpx1 activity, two traditional biomarkers of Se status over the range of Se deficient to eight times the Se requirement. Additional orthogonal biomarkers for supernutritional Se intakes and status, however, would further strengthen this approach. Such molecular biomarker panels would have the potential to better assess the boundary between safe but higher Se status and Se status indicative

of chronically toxic Se intake. Such biomarkers and the resulting panels would have high value in providing individual nutrient assessment, and such biomarkers/panels might even be able to differentiate between individuals that would benefit from Se supplementation versus being adversely affected.

ACKNOWLEDGEMENTS

This Research was supported in part by NIH Grant DK74184, and by the University of Wisconsin Agricultural Experiment Station grant WIS04909.

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(Received March 31, 2010, Accepted June 23, 2010)